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14. ABSTRACT

The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) in breast cancer cell motility, the phenotype critical for metastasis. The work I have conducted includes the demonstration that loss of PKD in a number of highly metastatic breast cancer cell lines results in a migration defect. I have also discovered a potential isoform specificity of PKD in the control of breast cancer cell motility.

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Introduction

The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) isoforms in breast cancer cell motility, the phenotype critical for metastasis. PKD is a serine/threonine kinase that has been best characterized as a key regulator of vesicular fission in golgi trafficking (1), a process is necessary for the delivery and secretion of molecules destined for the plasma membrane. This highlights its importance in homeostatic cell signaling, but despite four independent studies (2),(3),(4),(5) identifying upregulation and mutations of PKD in cancer tissue, a potential role for PKD in cancer progression has not been well explored. Based in part on a recent study which links PKD to fibroblast cell migration (6), I hypothesized that a PKD signaling network controls cytoskeletal reorganization and cellular adhesion, thereby regulating cell motility. I have tested the invasive migration of PKD1, PKD2, and PKD3-knockdown cells in transwell motility assays using 3T3-conditioned media as chemoattractant. I am exploring the specific mechanisms and signaling effectors that control this phenotype using mutant PKD constructs refractory to silencing by shRNA and a panel of both known and putative PKD substrates, mutated at known/putative sites of modification by. I have generating preliminary data which may result in the finding of a novel substrate of PKD. I will use live cell microscopy and immunofluorescence as additional methods to better elucidate the mechanism by which PKD controls invasive migration. This work will determine the regulation and function of a previously uncharacterized signaling pathway that is critical for breast cancer progression, the PKD signaling axis, and how it impacts invasive migration. The results of this research will yield an increased understanding of mechanisms that control the metastatic phenotype of breast carcinoma cells, subsequently allowing for new therapeutic strategies targeted to advanced stage tumors.

Body

The following tasks from the REVISED Statement of Work for this project were the focus for the research period from 30 September 2008-30 September 2009:

To investigate PKD isoform-specific mechanisms and substrates that control invasive migration in breast cancer cell lines by conducting migration defect rescue experiments (months 16-28).

b. Generate phospho-mimetic and non-phosphorylatable alleles of a panel of known and putative PKD substrates (months 22-24).

c. Analyze the ability of mutant alleles of both PKD1 and PKD2 that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites to rescue migration in PKD-knockdown cells (months 24-26).

d. Investigate the contribution of a variety of phospho-mimetic and non-phosphorylatable PKD substrates towards invasive migration in PKD1- and PKD2-knockdown cells (months 26-28).

To characterize the specific cytoskeletal changes that occur as a result of PKD loss using live cell microscopy (months 29-36).

a. Conduct time-lapse microscopy to compare efficiency of control and PKD-knockdown breast cancer cells to migrate (months 29-32).

b. Determine subcellular localization of PKD in breast cancer cell lines using GFP visualization and immunofluorescence (months 32-34).

c. Analyze specific cytoskeletal differences between control and PKD-knockdown migrating cells using time-lapse microscopy and immunofluorescent staining for cytoskeletal markers (months 34-36).

Progress

To investigate PKD isoform-specific mechanisms and substrates that control invasive migration in breast cancer cell lines by conducting migration defect rescue experiments

I have previously reported that PKD1 and PKD2 control cell migration in an isoform specific manner (**FIGURE 1**). However, the function of PKD3 remains unknown in this context. In my previous report, I provided preliminary RT-PCR data indicating that PKD3 is the most abundant isoform in most cancer cell lines tested. I have repeated this experiment and report the data below. (**FIGURE 2**) This surprising finding prompted me to analyze whether this isoform also controls breast cancer cell migration. The significance of such a finding would be a novel role for a relatively uncharacterized kinase. Importantly, either isoform redundancy or specificity could hamper the use of drugs to target these kinases in cancer therapy. Therefore, it is critical to obtain a comprehensive understanding of the function of all kinase family members in the control of this cellular behavior. Knockdown of PKD3 impairs cell migration in Sum159PT and MCF7 cells but not in MDA-MB-231 breast cancer cells (**FIGURE 3**). This result suggests that there may be some genetic discrepancies between these two breast cancer cell types that determine which signaling networks controls invasive migration. I have therefore completed a comprehensive analysis of the control of all PKD isoforms towards cellular migration in two breast cancer cell lines.

To investigate the role of PKD isoforms and specific PKD sites and domains, I am conducting migration defect rescue experiments. As reported previously, I generated mutant alleles of both PKD1 and PKD2 that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites. I developed a technique of depleting endogenous PKD and introducing mutant PKD alleles which involves the use of both lentiviral and retroviral infection. I introduced either vector

or the PKD non-silenceable rescue alleles retrovirally and selected a pool of positive infectants. This is followed by efficient knockdown of PKD isoforms using lentivirally delivered shRNAs that I have previously characterized. This method, compared to the more standard knockdown followed by transient transfection of PKD alleles, ensures viability of PKD-knockdown cells as well as efficient over-expression of re-introduced PKD alleles. Although I was able to achieve both PKD knockdown and over-expression of rescue constructs, I did not observe rescue of the migration defect caused by PKD knockdown (**FIGURE 4**). There are a few potential explanations for this result. PKD knockdown could cause irrevocable damage to the signaling networks and/or cellular mechanisms that control migration. Also, the differences between endogenous and re-introduced PKD could be responsible. Specifically, the tag on the rescue alleles could interfere with PKD function in this context. Also, the rescue alleles do not contain the untranslated regions of the gene that could be required for proper localization of PKD towards cell migration. To determine whether these latter scenarios could be the cause of the discrepancy, I obtained a full-length, untagged allele of PKD that I cloned into a mammalian expression vector. I am currently conducting experiments using this construct to determine whether it rescues the migration defect of breast cancer cells. If I am able to achieve rescue using this PKD construct, I will generate mutations in domains and phosphorylation sites of interest to determine their contribution towards cell migration.

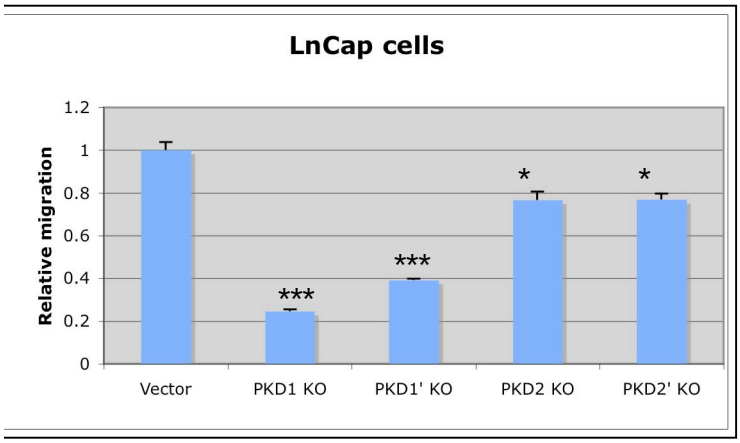
I am also investigating the contribution of a variety of both known and putative PKD substrates and effectors in PKD isoform signaling networks towards invasive migration. This panel, most of which I have already generated, includes phospho-mimetic and non-phosphorylatable alleles of RIN1, HSP27, and PI4KIIIβ, all of which are known PKD substrates and similarly mutated alleles of B-Raf, Rac1 (a member of the Rho family of GTPases), and Rabaptin-5 (an effector of Rab-5), all of which are putative PKD substrates.

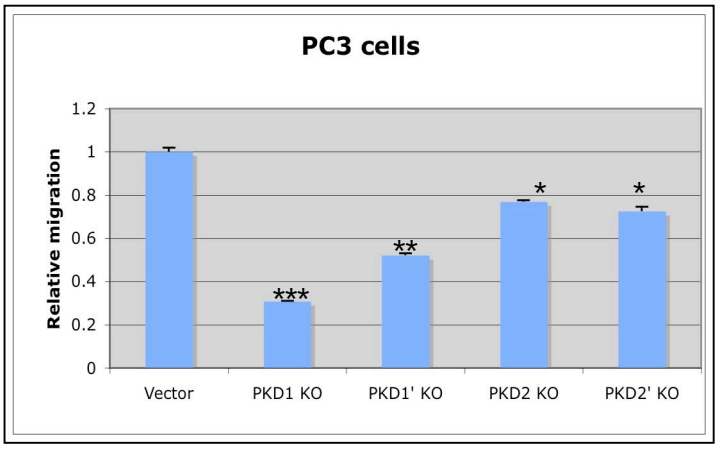
I have focused a significant portion of my recent efforts towards the identification of a new putative substrate of PKD, Rabaptin-5. An unpublished large-scale mass spectrometry study from Cell Signaling Technologies isolated a phosphorylated peptide of Rabaptin-5 that represents the preferred amino acid motif of PKD substrates. The phosphorylation status of this motif is significantly affected by the use of a number of cancer drugs and tumor-promoting kinase inhibitors in breast cancer cell lines. Rabaptin-5 is an effector of Rab-5, which controls trafficking of endocytotic vesicles, a function closely linked to PKD's role in the trafficking of golgi vesicles. Rabaptin-5 has also been found to localize to the golgi membrane and has been implicated in cell migration (7,8). This suggests the exciting possibility that PKD regulates Rabaptin-5 in the control of cell motility. I have first sought to identify whether Rabaptin-5 is phosphorylated by PKD. I treated cells expressing vector, Myc-tagged-Rabaptin-5, or PKD1 as a positive control (PKD autophosphorylates) with PDBu, a phorbol ester that is a potent activator of PKD. I then probed the lysates of these cells using an antibody directed at phosphorylated PKD substrates (αphospho-PKD-substrate MOTIF) and observe a band that migrates at the expected size of Rabaptin-5 (**FIGURE 5**). Additionally, I co-transfected cells with Rabaptin-5 and either wild type or mutant PKD alleles to determine the contribution of PKD activity to Rabaptin-5 phosphorylation as detected by the phospho-PKD-MOTIF antibody. Wild type and constitutively active alleles of PKD result in Rabaptin-5 phosphorylation while kinase inactive PKD or vector alone do not. These blots were also probed with a Myc antibody to detect expression of Rabaptin-5. These preliminary results suggest that Rabaptin-5 is a PKD substrate. I am currently testing the functional significance of this novel signaling network.

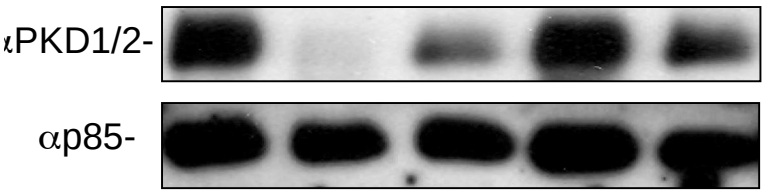
To characterize the specific cytoskeletal changes that occur as a result of PKD loss using live cell microscopy

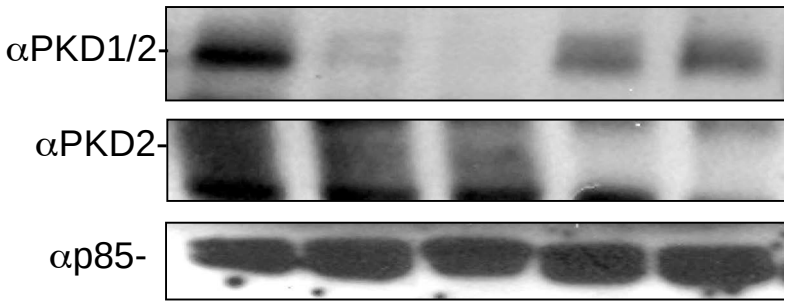
My focus on the above and further investigation of exciting and unexpected results has prevented me from performing these experiments. I intend to complete them within the next 6 months to be included in the manuscript I am developing.

FIGURE 1 **PROSTATE CANCER CELL LINES**

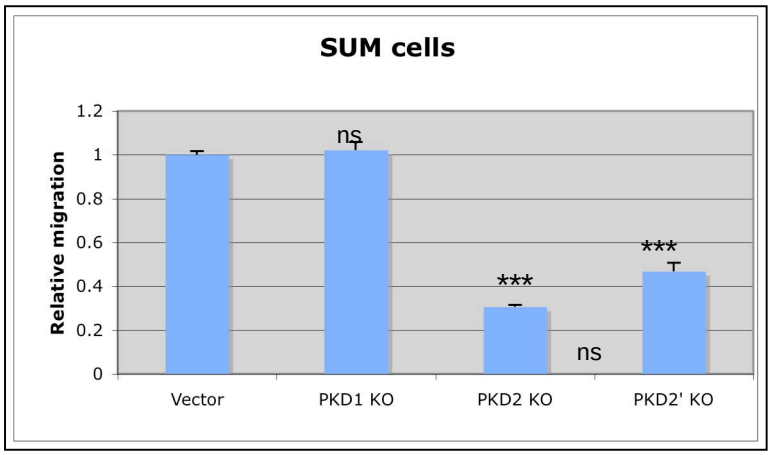


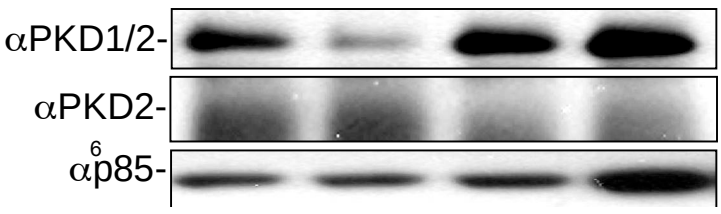


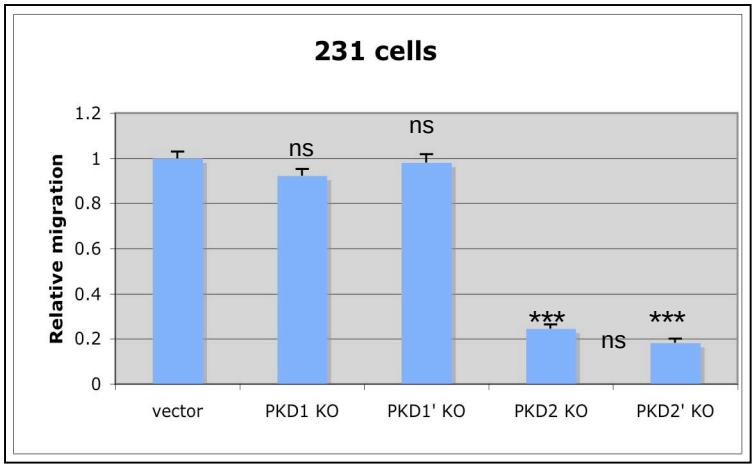


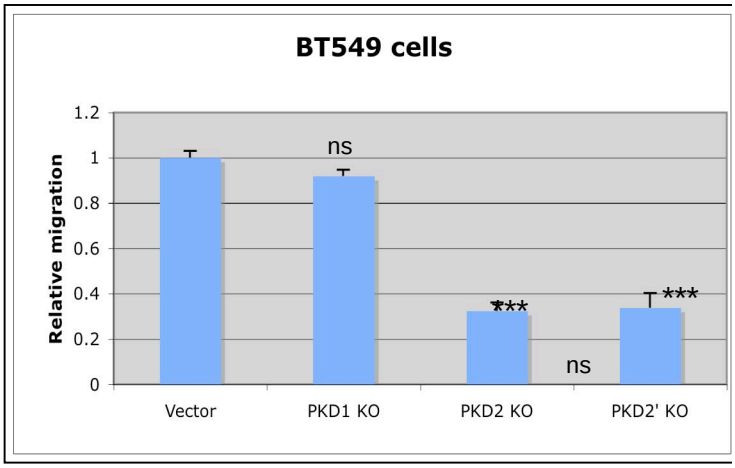


BREAST CANCER CELL LINES









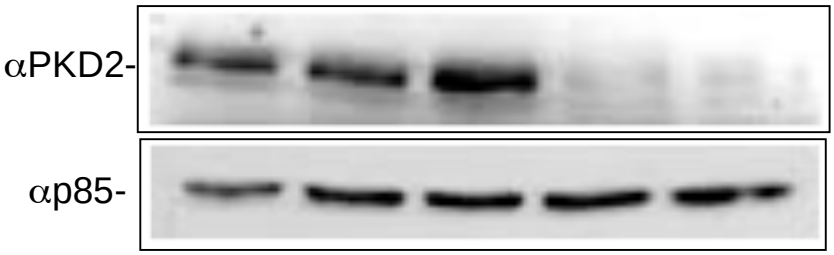
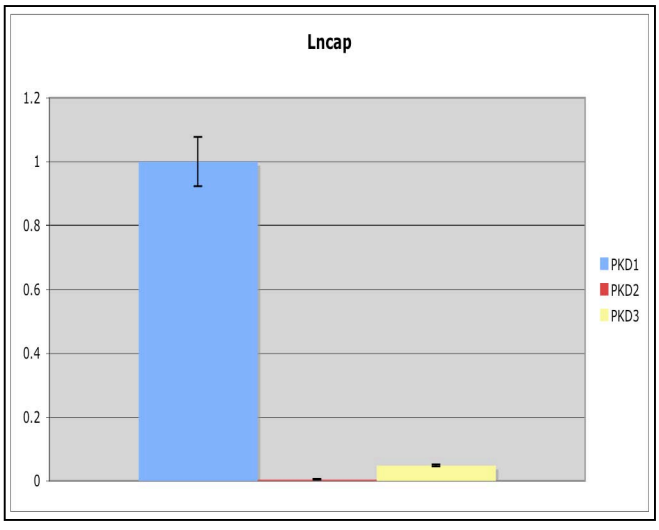
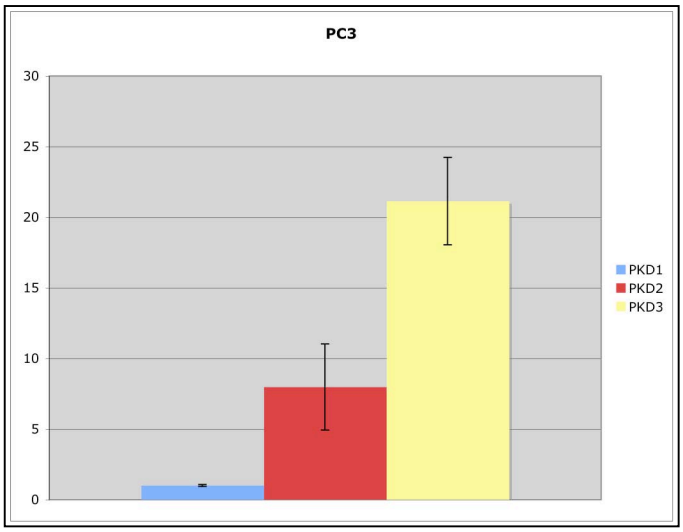
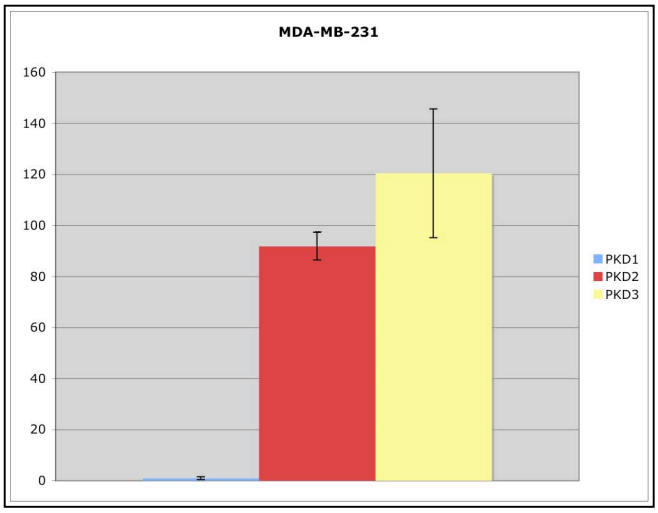
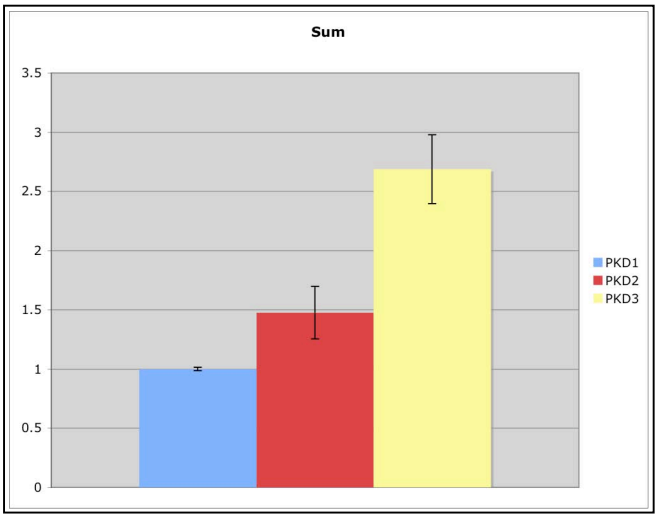


FIGURE 2: RT-PCR






MDA-MB-231
160
140
120
100
80
60
40
20
0
PKD1
PKD2
PKD3


Sum
3.5
3
2.5
2
1.5
1
0.5
0
PKD1
PKD2
PKD3

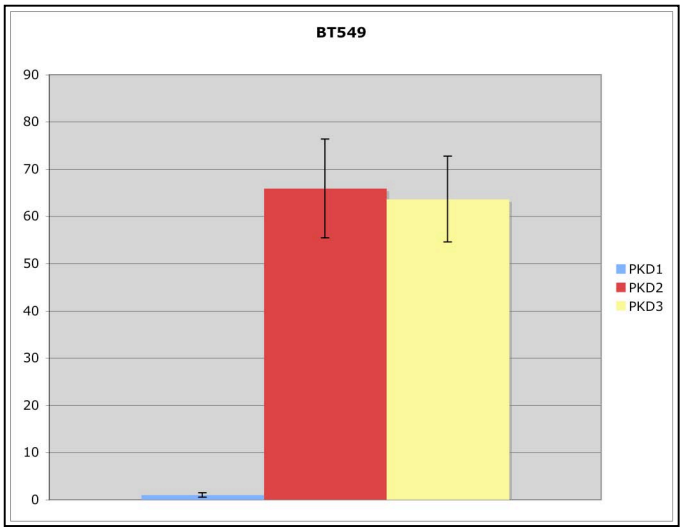

BT549
90
80
70
60
50
40
30
20
10
0
PKD1
PKD2
PKD3

FIGURE 3. Knockdown of PKD3 impairs cell migration

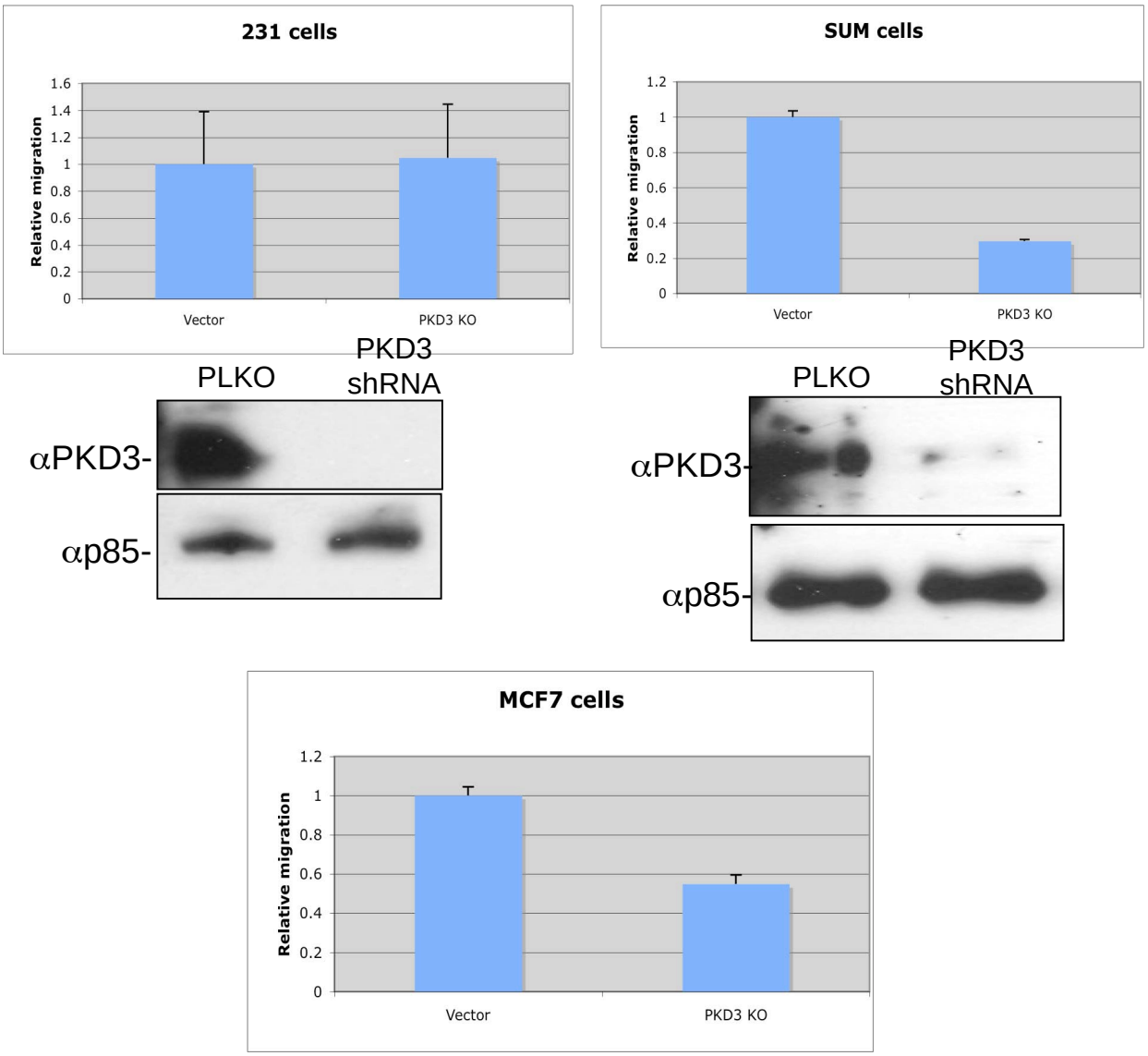


FIGURE 4. Reintroduction of PKD2 does not rescue the migration defect of knockdown cells

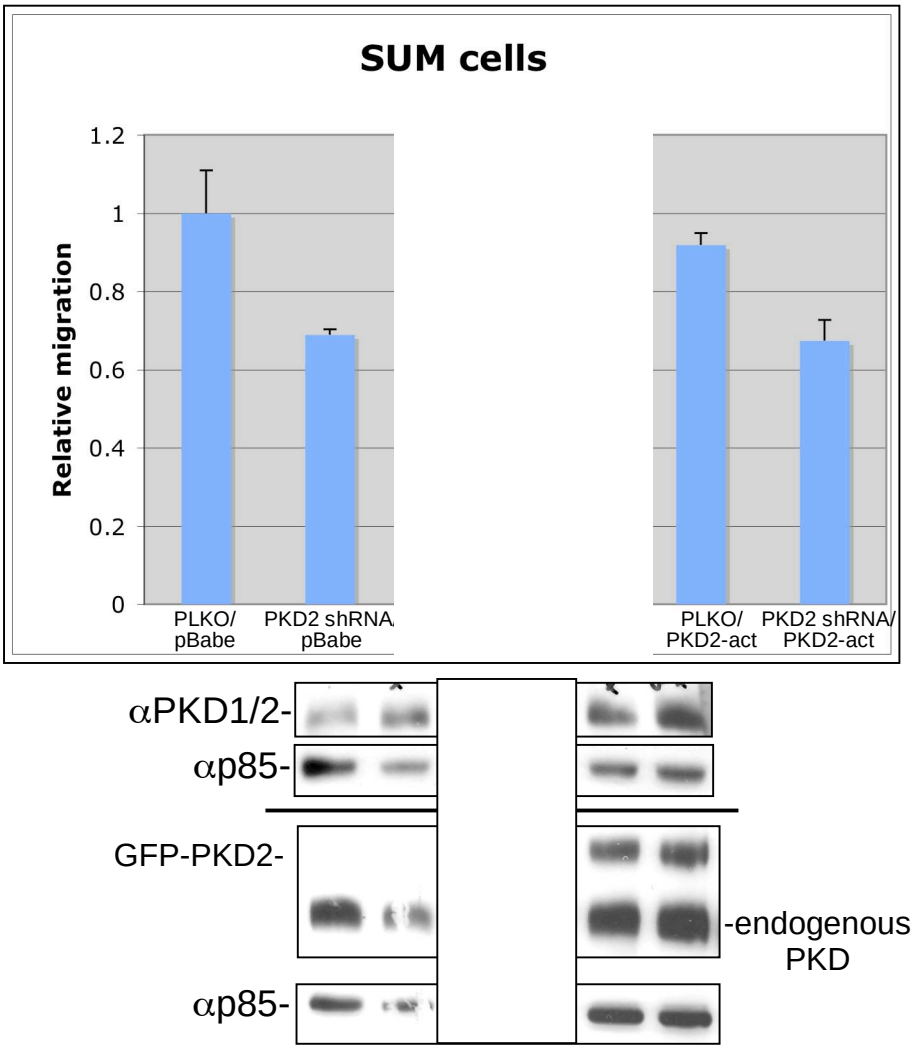
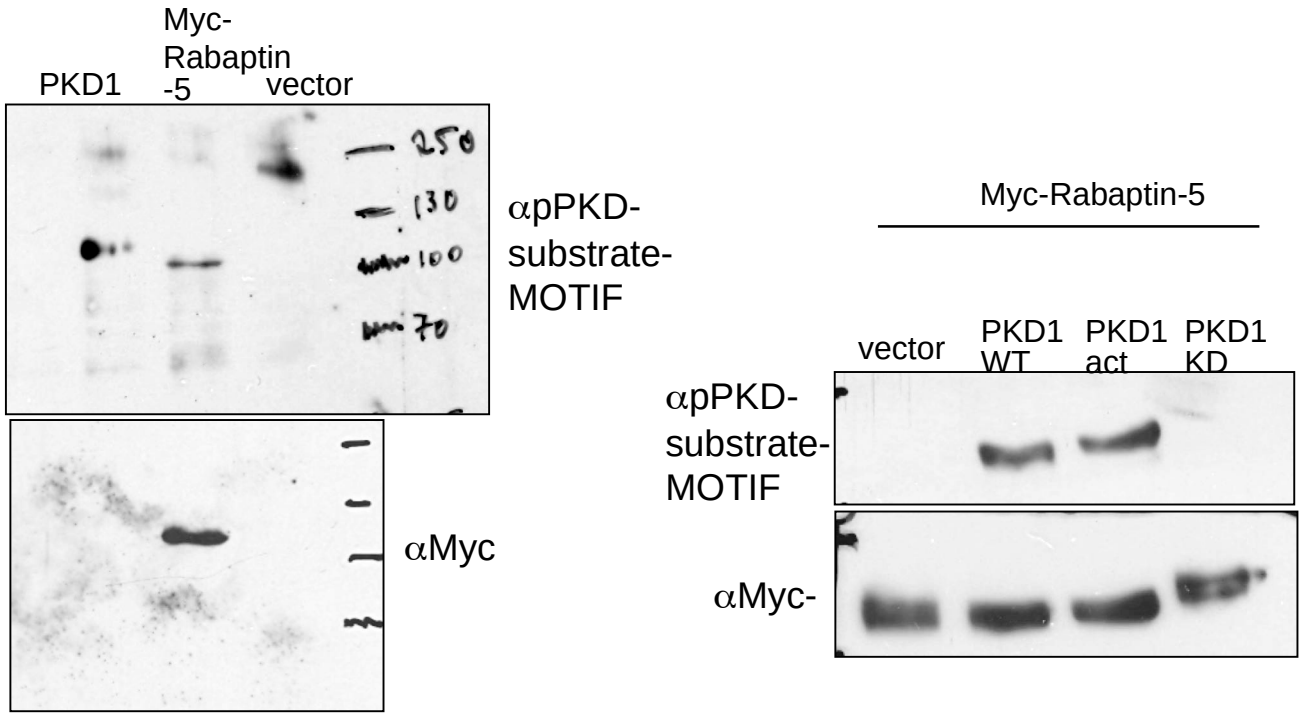


FIGURE 5. Rabaptin-5 is a putative PKD substrate



Current research and future directions

As a result of the discovery that PKD controls cell migration in an isoform-specific manner, I have sought to develop a high-impact study demonstrating a role for PKD isoforms in the control of breast cancer cell migration and delineating the specific mechanisms by which PKD isoforms and their signaling substrates that control this phenotype. I intend for the manuscript I am developing to encompass a comprehensive study demonstrating first the migration-defect phenotype I observe for PKD-knockdown cells and then describing the specific signaling mechanisms by which PKD isoforms control this phenotype. This section will optimally include PKD mutant allele rescue analysis to determine which PKD domains and phosphorylation sites are responsible for this phenotype. Therefore, a primary ongoing effort is to repeat the rescue experiments using a full-length, un-tagged PKD that is refractory to silencing by shRNA. I would also like this section to highlight the role(s) of PKD substrates and effectors that control cell migrations. I therefore am testing the possibility that PKD signals through Rabaptin-5 to control cell migration. I am analyzing the phosphorylation of Rabaptin-5 (using the PKD-substrate-directed antibody) in cells in which PKD is present or depleted and in which PKD is stimulated or overepressed. I am also generating both non-phosphorylatable and phospho-mimetic alleles of Rabaptin-5 to determine the functional relevance of PKD-phosphorylation at this site. Whether Rabaptin-5 mediates signals by which PKD controls cell migration or not, the discovery that it is a PKD substrate would be a novel and important finding worthy of further investigation. And finally, I would like to include live cell microscopy and immuno-fluorescence analyses to determine both the localization of PKD and the specific morphological defects that account for the impaired migration phenotype.

Key Research Accomplishments and Reportable Outcomes

- **Completed a comprehensive analysis of the control of PKD isoforms towards cellular migration in two breast cancer cell lines, including the relatively uncharacterized PKD3 isoform.**
- **Successfully achieved PKD knockdown/over-expression experiments although, unexpectedly, rescue of the migration defect caused by PKD knockdown was not observed.**
- **Discovered a putative substrate of PKD, Rabaptin-5, which would represent the first link between PKD and the control of endocytosis, and which could be mediating PKD-controlled invasive migration.**

Conclusion

The overarching goal of my project as outlined in the original application has been to analyze the role of Protein Kinase D (PKD) isoforms in breast cancer cell motility, the phenotype critical for metastasis. In my first year of study I optimized both overexpression and silencing cell infection systems with which to conduct gain and loss of function experiments using breast cancer cell lines in invasive migration transwell assays. I also worked in collaboration with CRT to develop and test a panel of PKD-specific inhibitors. I was thereby able to determine a reproducible migration-defect phenotype of PKD-knockdown and inhibition in HS578T cells, which are a highly metastatic breast cancer cell line. I also observed a loss on cellular viability caused by PKD depletion which, by means of thorough Propidium Iodide-FACS analysis I found to be an artifact caused by use of a first generation PKD

inhibitor. This allows me to conclude that the migration-defect phenotype I observe of PKD-knockdown cells is not due to a general viability issue but instead due to the impairment of the specific signaling networks controlled by PKD.

In the following year of study, I demonstrated that PKD loss by lentiviral shRNA knockdown results in a migration defect phenotype in a number of metastatic breast and prostate cancer cell lines. I further demonstrated that the PKD2 signaling network is dominant in the control of this phenotype in breast cancer cell types Sum159PT and BT549. I confirmed by RT-PCR that both isoforms are present in significant quantities in at least one of these cell types and therefore discovered a potential isoform specificity of PKD in the control of breast cancer cell motility. As a result of this discovery, I decided to broaden the scope of my studies to focus on isoform specific mechanisms and a number of potential substrates that control this phenotype. I therefore created a number of PKD1 and 2 non-silenceable and PKD substrate mutant alleles for PKD-knockdown/rescue analysis.

In my final year of study I sought to gain insights about the signaling and mechanisms of PKD-controlled breast cancer cell migration using these mutant alleles in the transwell migration assay. I developed an efficient PKD knockdown/ over-expression system for use in the transwell migration assays but unexpectedly did not observe rescue of the migration defect caused by PKD knockdown. This is likely either due to irrevocable damage to the migration machinery caused by PKD or because of the differences between endogenous and re-introduced PKD. Prompted by my RT-PCR data indicating that PKD3 is the most abundant isoform in most cancer cell lines tested, I began to test the role of this less characterized isoform in breast cancer cell migration. Knockdown of PKD3 either using a number of specific shRNAs together or individually significantly impairs cell migration in Sum159PT but not MDA-MB-231 breast cancer cells indicating that there may be some genetic discrepancies between these two breast

cancer cell types that determine which signaling networks controls invasive migration. I have therefore completed a comprehensive analysis of the control of all PKD isoforms towards cellular migration in two breast cancer cell lines.

Additionally, I have recently focused on the identification of signaling effectors of PKD in the control of cell migration. I have discovered a putative signaling substrate of PKD, Rabaptin-5, which would represent the first link between PKD and the control of endocytosis, and which could be mediating PKD-controlled invasive migration.

To summarize, I have demonstrated that PKD2 and PKD3 isoforms are required for cell migration in breast cancer cell lines while, unexpectedly, PKD1, the best-characterized isoform, is not. I developed both a panel of mutant PKD alleles for use and an efficient PKD knockdown/ over-expression system to gain better insights into the signaling mechanisms by which PKD isoforms control cell migration. Either because PKD knockdown causes irrevocable damage to the migration machinery or because of the differences between endogenous and re-introduced PKD I did not observe rescue of the migration defect caused by PKD knockdown. Furthermore, I have provided preliminary evidence that Rabaptin-5, a Rab-5 effector in endocytosis, is a substrate of PKD. These findings are novel and provide exciting possibilities for signaling mechanisms by which PKD controls breast cancer cell migration.

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